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PRINCIPAL INVESTIGATOR: Christopher P. Baines, Ph.D.

CONTRACTING ORGANIZATION: University of Missouri
Columbia, MO 65211-3020

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**14. ABSTRACT**
The planned research proposes to examine the ability of inhibition of mitophagy, the mitochondrial-specific form of autophagy, to kill prostate cancer cells. Cancer cells become increasingly dependent on mitophagy as an energy source and the mitochondria themselves become more and more dysfunctional. Therefore, mitophagy may represent a novel target for the prevention of prostate cancer progression. Consequently, the *purpose* of this research is to test whether inhibition of mitophagy can lead to the death of prostate cancer cells. Key mediators of the mitophagic process, specifically Parkin, dynamin-related protein-1 (Drp1), fission-1 (Fis1), and cyclophilin-D (CypD), will be genetically disrupted by shRNA. The effects that mitophagy blockade has on mitochondrial function, ROS production and ultimately survival of normal prostate cells as well as several different prostate cancer cell lines, especially highly aggressive cells, will then be examined. We experienced considerable problems with the shRNA approach but have successfully circumvented this using siRNAs, such that we now have efficient knockdown of each protein in DU145, LNCaP and PC3 prostate cancer cells. Knockdown of CypD beneficially affected mitochondrial function and reduced reactive oxygen species production (ROS), suggesting that CypD may not be a viable target for the treatment of prostate cancer. In contrast, targeting of Fis1 and especially Drp1 maybe of therapeutic benefit as they were found to induce mitochondrial dysfunction and/or ROS production along with a compensatory increase in general autophagy. We are currently recapitulating these studies in the Parkin-deficient prostate cancer cells and will then test whether the disruption of Fis1, Drp1 and Parkin can sensitize the prostate cancer cells to paclitaxel exposure. Such a finding would be of huge significance as this knowledge could be used to generate inhibitors of Fis1, Drp1, and/or Parkin for the use as adjunct therapies alongside conventional chemotherapeutic interventions to better treat prostate cancers.

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Prostate cancer, Mitochondria, Mitophagy, Reactive oxygen species, Cell death

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## Introduction

As a cancer cell progresses down the path of malignancy, it becomes more reliant upon autophagy as its main source of energy required for survival, proliferation, and migration (1). In particular, mitophagy - the selective removal of mitochondria - is critical for the continued survival of the cancer cell (2). Mitochondria from prostate cancer cells are more dysfunctional than those in normal cells in that they exhibit more mtDNA mutations and enhanced reactive oxygen species (ROS) production (3). Moreover, autophagy, and by inference mitophagy, enables prostate cancer cells to survive androgen deprivation (4). Therefore, mitophagy may represent a novel target for the prevention of prostate cancer progression and the development of anti-androgen resistance. *Thus, the current hypothesis is that the specific inhibition of mitophagy in prostate cancer cells will lead to cell death by 2 distinct mechanisms:* 1) by cutting off the cancer cell's main energy supply, and 2) by causing the accumulation of toxic dysfunctional mitochondria. Consequently, the *purpose* of this research is to test whether inhibition of mitophagy can lead to the death of prostate cancer cells. Key mediators of the mitophagic process, specifically Parkin, dynamin-related protein-1 (Drp1), fission-1 (Fis1), and cyclophilin-D (CypD)(5), will be genetically disrupted by shRNA. The effects that mitophagy blockade has on mitochondrial function, ROS production and ultimately survival of normal prostate cells as well as several different prostate cancer cell lines, especially highly aggressive cells, will then be examined.

## Body

For the this report I will go through the research accomplishments as they relate to each Task, as outlined in the Statement of Work.

### Task 1: Identify shRNAs that can effectively knockdown Parkin, Fis1, Drp1, and CypD both the normal and cancer prostate cell lines.

This initial step has proven to be the most problematic. We obtained several shRN-containing plasmids for each targeted protein and systematically began transfecting them in the various prostate cancer cell lines to test their effectiveness in knocking down the protein. However, we did not see any major knockdown of any of the proteins. We were concerned that this was due to our transfection efficiency and ran control experiments with a GFP-containing plasmid – indeed our efficiency was not very high (10-20%). We then picked one set of shRNAs (CypD) and tested them with various transfection reagents. However, we still did not see any knockdown of the protein even when we selected for the plasmid. Due to this constant testing and re-testing we lost several months simply trying to get something to work.

When it became apparent that we were not succeeding with the shRNA approach, we decided to switch instead to simple siRNAs which are easier to transfect into cells. This has proved extremely successful and we have achieved significant knockdown of all 4 proteins (CypD, Fis1, Drp1, and Parkin) in the DU145, LNCaP and PC3 prostate cancer cell lines. As an example, **Figure 1** shows the efficient knockdown of CypD, Fis1, Drp1 and Parkin in the DU145 cells. Initial studies indicate these siRNAs are also effective in the RWPE1 control line. Thus we have, for all intense purposes, completed Task 1.

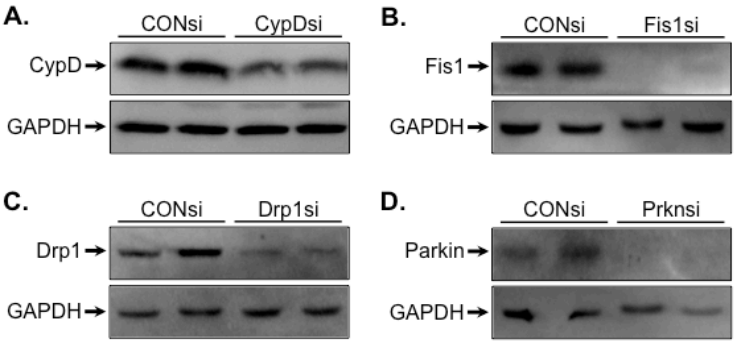


**Figure 1. Effectiveness of siRNA knockdown of CypD, Fis1, Drp1, and Parkin in DU145 cells.** DU145 cells were transfected with either a control siRNA or specific siRNAs targeting (**A**) Cypd, (**B**) Fis1, (**C**), Drp1, and (**D**) Parkin. After 48 hrs the cells were harvested, lysed, and then subjected to immunoblotting for the respective protein. GAPDH was used as loading control.

**Task 2: Examine the effects of Parkin, Fis1, Drp1, and CypD knockdown on mitochondrial function in the normal and cancer prostate cell lines.**

Having now established effective siRNAs against each protein we have been assessing the effect of their knockdown on mitochondrial function in the various prostate cancer cell lines. We first assessed the effects of protein depletion on mitochondrial membrane potential and reactive oxygen species (ROS) production in the prostate cancer cell lines using TMRE and DCF fluorescence, respectively. For example, we achieved a roughly similar knockdown of CypD protein between the DU145, LNCaP and PC3 cell lines (**Figure 2**). This consistently led to an increase in TMRE fluorescence (**Figure 3**), regardless of the cell line suggesting a better respiratory function in these cells. Consist with this we saw a decrease in basal ROS production in all 3 cell lines (**Figure 4**). This indicates that removal of CypD may actually be beneficial for the cancer cells, consistant with CypD's role as a critical regulator of the mitochondrial permeability transition pore (6), opening of which causes mitochondrial dysfunction, rather than of mitophagy (7).

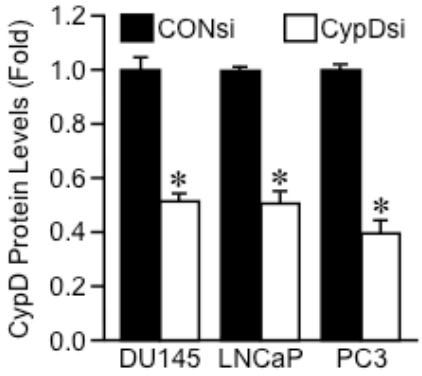


**Figure 2. Knockdown of CypD in prostate cancer cells.** DU145, LNCaP, and PC3 cells were transfected with a control or CypD-specific siRNA for 48 hrs followed by immunoblotting for CypD. **P*<0.05 vs. CONsi.

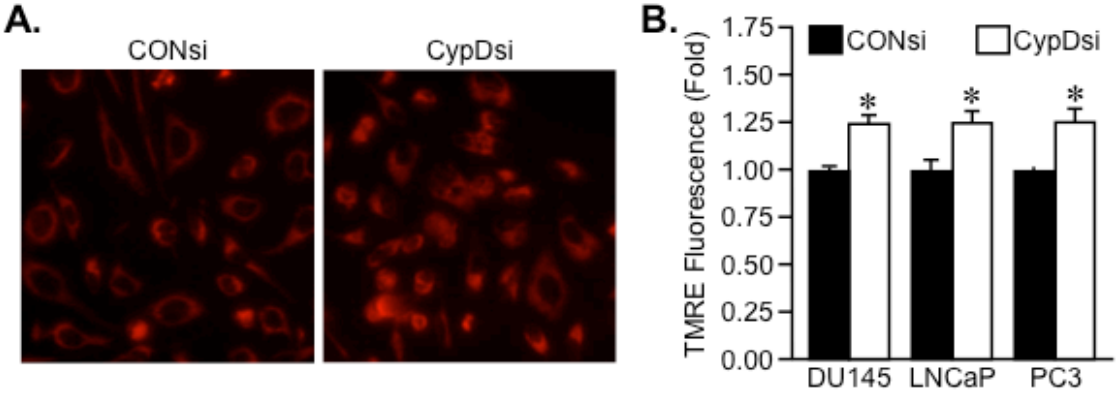


**Figure 3. Knockdown of CypD increases mitochondrial potential in prostate cancer cells.** (**A**) DU145 cells were transfected with a control or CypD-specific siRNA for 48 hrs and then stained with TMRE. (B) Quantification of TMRE fluorescence in DU145, LNCaP, and PC3 cells. **P*<0.05 vs. CONsi.

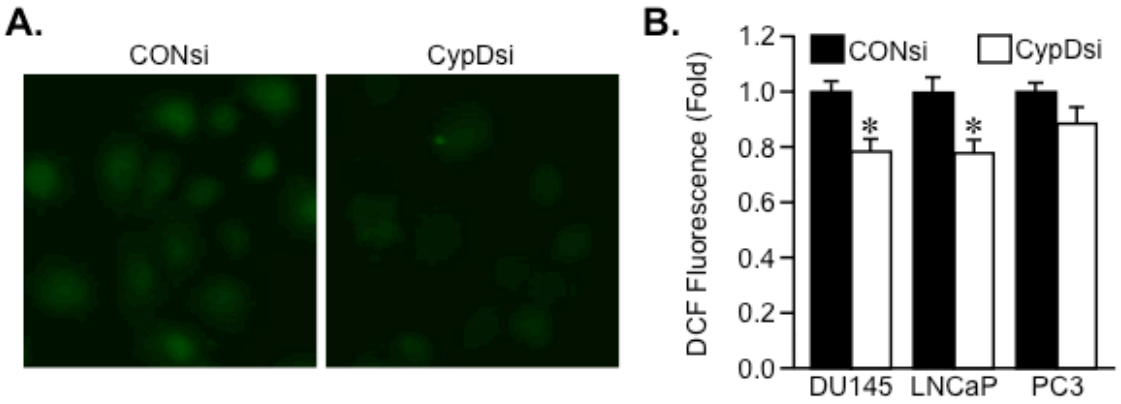


**Figure 4. Knockdown of CypD decreases ROS production in prostate cancer cells.** (**A**) DU145 cells were transfected with a control or CypD-specific siRNA for 48 hrs and then stained with DCF. (**B**) Quantification of DCF fluorescence in DU145, LNCaP, and PC3 cells. **P*<0.05 vs. CONSi.

We have also conducted these studies in the Fis1 deficient cells. We have achieved a significant knockdown of Fis1 in the 3 different cancer lines, especially in the DU145 cells (**Figure 5A**). However, unlike with the CypD siRNA, Fis1 knockdown did not affect mitochondrial membrane potential (**Figure 5B**). However, we did see an increase in ROS production in the Fis1-depleted PC3 cells, but not the DU145 cells (**Figure 5C**), suggesting a cancer stage-specific effect (see also the LC3

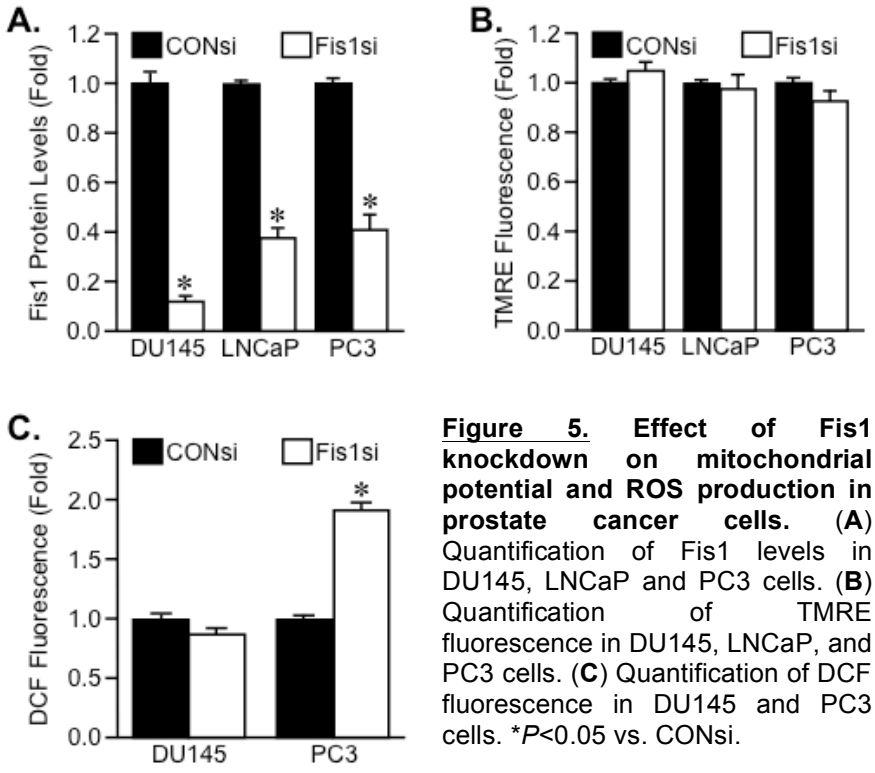


**Figure 5. Effect of Fis1 knockdown on mitochondrial potential and ROS production in prostate cancer cells.** (**A**) Quantification of Fis1 levels in DU145, LNCaP and PC3 cells. (**B**) Quantification of TMRE fluorescence in DU145, LNCaP, and PC3 cells. (**C**) Quantification of DCF fluorescence in DU145 and PC3 cells. **P*<0.05 vs. CONSi.

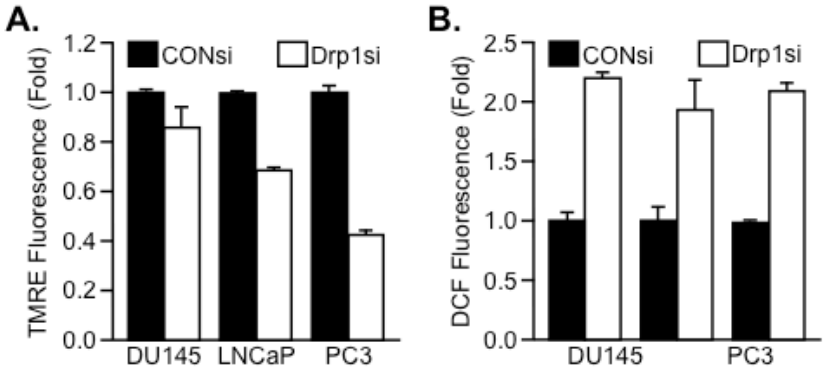


**Figure 6. Effect of Drp1 knockdown on mitochondrial potential and ROS production in prostate cancer cells.** (**A**) Quantification of TMRE fluorescence in DU145, LNCaP, and PC3 cells. (**B**) Quantification of DCF fluorescence in DU145, LNCaP and PC3 cells. **P*<0.05 vs. CONSi.

response in Task 3). We also have preliminary data from the Drp1 knockdown cells. Here we see a decrease in mitochondrial membrane potential coupled with an increase in ROS production (**Figure 6**). We are currently finishing up the Drp1 studies. Together, this suggests that Fis1 and especially Drp1 depletion leads to the build up of more dysfunctional, ROS producing mitochondria. This is in line with our hypothesis and has been reported for Drp1 inhibition in HeLa cells (8).

In addition to measuring mitochondrial function another goal was to assess the effects of the various knockdowns on the major proteins in the electron transport train. As an example we have measured the levels of the alpha subunit of the ATP mitochondrial ATP synthase. Knockdown of CypD had no effect whatsoever on levels of the ATP synthase subunit in the DU145 cells (**Figure 7A**). In contrast both Fis1 and Drp1 depletion significantly reduced ATP synthase levels in DU145s (**Figure 7B**). We have observed a similar effect in the LNCaP and PC3 cells as well. This is consistent with the mitochondrial dysfunction/ROS production we see with the Fis1 and Drp1 knockdowns. We are now embarking on characterizing the Parkin deficient cells as well as testing the effects of these knockdowns on mitochondrial function and protein expression in the normal prostate cell line.

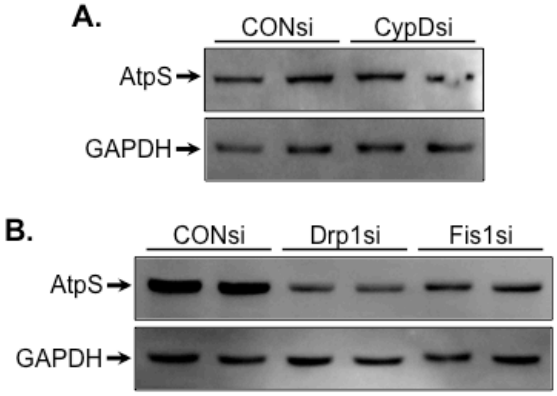


**Figure 7. Effect of CypD, Fis1, and Drp1 knockdown on ATP synthase expression.** (**A**) DU145 cells were transfected with a control or CypD-specific siRNA for 48 hrs and then immunoblotted for ATP synthase (AtpS). (**B**) DU145 cells were transfected with a control siRNA or Drp1- or Fis1-CypD-specific siRNA for 48 hrs and then immunoblotted for AtpS. GAPDH was used as a loading control.

**Task 3: Examine the effects of Parkin, Fis1, Drp1, and CypD knockdown on autophagy/mitophagy in the normal and cancer prostate cell lines.**

We have also been assessing the effects of each knockdown on autophagy using the GFP-LC3 reporter. Induction of autophagy in the cells leads to the generation of GFP-LC3 puncta due to autophagosome formation, with a certain number of cells exhibiting these at baseline (**Figure 8A**). Knockdown of CypD did not significantly affect the number of puncta-containing cells in either the DU145 or the PC3 lines (**Figure 8A,B**), suggesting the level of autophagy was unchanged in the

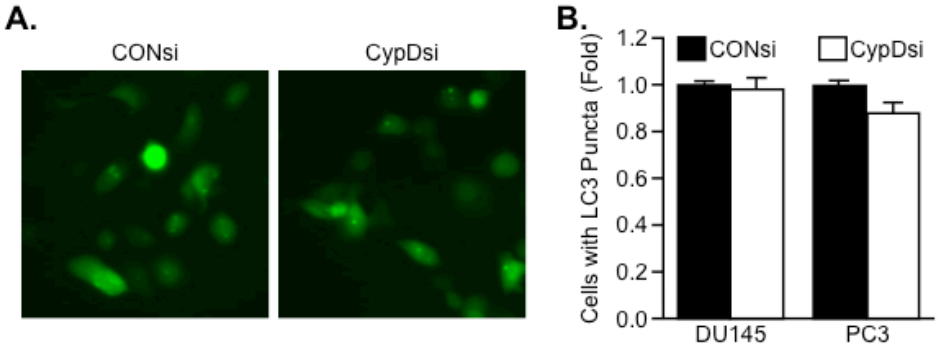


**Figure 7. Effect of CypD knockdown on GFP-LC3 puncta formation.** (**A**) DU145 cells were transfected with a control or CypD-specific siRNA for 48 hrs and the infected with an adenovirus encoding GFP-LC3. (**B**) Quantification of the number of puncta-containing cells in the control and CypD siRNA-transfected DU145 and PC3 cell lines.

cells. In contrast, Fis1 knockdown greatly elevated the level of autophagy in the PC3 cells (**Figure 9A**) – an effect that was consistent with increase in ROS seen in these cells (see **Figure 5C**), suggesting a link between the two phenomena. This potential link was further emphasized by the fact that autophagy was increased in both DU145 and PC3 cells upon Drp1 depletion (**Figure 9B**), again concomitant with increases in ROS production. Thus general autophagy appears to be elevated when ROS production is increased. Consistent with increases in autophagy seen in the PC3 cells, we observed a substantial increase in the expression of beclin-1, a major mediator of autophagy in the Fis1 and Drp1 depleted PC3s (**Figure 10**). Knockdown of Parkin had a similar effect (**Figure 10**). These increases in general autophagy maybe a compensatory mechanism for a decrease in mitophagy. Indeed, we are now testing the levels of mitophagy in these cells.

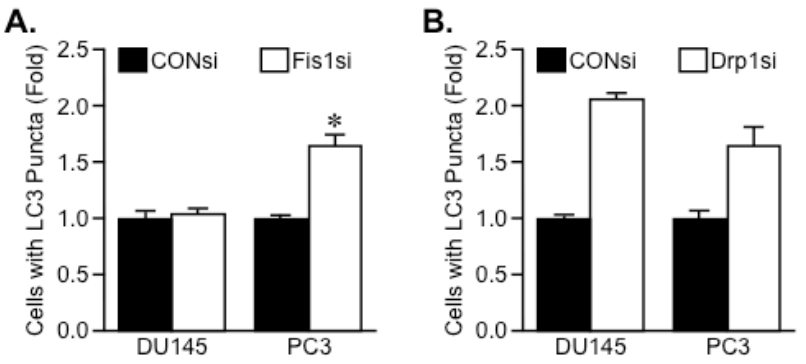


**Figure 9. Effect of Fis1 and Drp1 knockdown on GFP-LC3 puncta formation.** (**A**) Quantification of the number of puncta-containing cells in control and Fis1 siRNA-transfected DU145 and PC3 cell lines. (**B**) Quantification of the number of puncta-containing cells in control and Drp1 siRNA-transfected DU145 and PC3 cell lines.

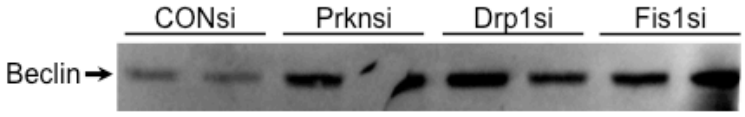


**Figure 10. Effect of Parkin, Drp1, and Fis1 knockdown on Beclin expression.** PC3 cells were transfected with control, Parkin, Fis1, or Drp1 siRNAs for 48 hrs and then immune-blotted for Beclin.

**Task 4: Examine the effects of Parkin, Fis1, Drp1, and CypD knockdown on cell death in the normal and cancer prostate cell lines.**

We have focused our efforts on the first 3 tasks. As we complete the other tasks we will shift our focus to these key experiments.

## Key Research Accomplishments

- Identified and characterized siRNAs that efficiently knockdown CypD, Fis1, Drp1, and Parkin
- Found that CypD depletion beneficially, rather than detrimentally, affects mitochondrial function and ROS production
- Demonstrated that Fis1 knockdown selectively upregulates ROS production and autophagy in PC3 prostate cancer cells
- Demonstrated that Drp1 depletion reduces mitochondrial function, upregulates ROS production and autophagy in all 3 prostate cancer cell lines
- Shown that induction of general autophagy by Fis1 and Drp1 is associated with increased ROS production and enhanced levels of the autophagic mediator Beclin-1

## Reportable Outcomes

None as of yet. But we hope to submit a manuscript as well as applications for future funding in the upcoming year.

## Conclusion

In summary, we have made significant progress towards the completion of the proposed work. In particular our data so far indicates that inhibition of CypD may not be a viable target for the treatment of prostate cancer cells due to the beneficial effects on mitochondrial function. In contrast, targeting of Fis1 and especially Drp1 maybe of therapeutic benefit as they induce mitochondrial dysfunction and/or ROS production which would set the cancer cell up for further cytotoxic interventions. We expect similar results with our Parkin knockdown. Our goal for the coming year is to complete the various mitochondrial and autophagic studies and most importantly test whether inhibition of the mitophagic mediators sensitizes the prostate cancer cells to paclitaxel. Should this be the case then this knowledge could be used to generate inhibitors of Fis1, Drp1, and/or Parkin for the use as adjunct therapies alongside conventional chemotherapeutic interventions to better treat prostate cancers.

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